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Methionine sulfoxide reductases protect Ffh from oxidative damages in *Escherichia coli*

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In proteins, methionine residues are primary targets for oxidation. Methionine oxidation is reversed by methionine sulfoxide reductases A and B, a class of highly conserved enzymes. Ffh protein, a component of the ubiquitous signal recognition particle, contains a methionine-rich domain, interacting with a small 4.5S RNA. *In vitro* analyses reported here show that: (i) oxidized Ffh is unable to bind 4.5S RNA, (ii) oxidized Ffh contains methionine sulfoxide residues, (iii) oxidized Ffh is a substrate for MsrA and MsrB enzymes; and (iv) MsrA/B repairing activities allow oxidized Ffh to recover 4.5S RNA-binding abilities. *In vivo* analyses reveal that: (i) Ffh synthesized in the *msrA msrB* mutant contains methionine sulfoxide residues and is unstable, (ii) *msrA msrB* mutant requires high levels of Ffh synthesis for growth and (iii) *msrA msrB* mutation leads to defects in Ffh-dependent targeting of MalF. We conclude that MsrA and MsrB are required to repair Ffh oxidized by reactive oxygen species produced by aerobic metabolism, establishing an as-yet undescribed link between protein targeting and oxidation.

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Introduction

The functional alteration of proteins by oxidation emerges as a central cause of various pathologic disorders (Stadtman and Levine, 2000). Reactive oxygen species (ROS) are produced by aerobic metabolism or under specific conditions such as host:parasite interactions and aging process (Stadtman, 1992). The frequent targets of oxidation in proteins are methionine (Met) residues, which are converted to methionine sulfoxide (MetSO) (Vogt, 1995). Methionine sulfoxide reductase A (MsrA), which regenerates Met from MetSO, occurs in most living cells (Brot *et al*, 1981). The ubiquitous distribution of *msrA* was expected to correlate with an essential function in cellular physiology. Accordingly, alteration of MsrA synthesis caused global phenotypes such as modified life span in mouse and drosophila (Moskovitz *et al*, 2001; Ruan *et al*, 2002), attenuated virulence in bacterial pathogens (Wizemann *et al*, 1996; Hassouni *et al*, 1999) or increased sensitivity to a high concentration of exogenously added oxidative agents (Moskovitz *et al*, 1995, 1998). Surprisingly, however, no major physiological defects were observed in an *Escherichia coli msrA* mutant grown under normal aerobic growth conditions (Moskovitz *et al*, 1995; our unpublished data).

Recently, we identified a second ubiquitous methionine sulfoxide reductase encoding gene, referred to as *msrB* (Grimaud *et al*, 2001; Kryukov *et al*, 2002; Olry *et al*, 2002). Biochemical investigations established that MsrA and MsrB enzymes exhibit different diastereospecificity, the former acting on Met-(S)-SO and the latter on Met-(R)-SO diastereoisomers (Sharov *et al*, 1999; Kryukov *et al*, 2002; Olry *et al*, 2002). This diastereospecificity accounted for the fact that MsrA and MsrB enzymes were required for reduction of all MetSO present in oxidized calmodulin, while the individual action of each allowed reduction of a subset of MetSO only (Grimaud *et al*, 2001). No drastic phenotypic changes were found to be associated with *msrB* mutation in *E. coli* (Grimaud *et al*, 2001).

In order to investigate the physiological role of the MsrA/B repair pathway *in vivo*, our strategy was to identify a protein with the following criteria: (i) the function of this protein should be associated with easily scorable phenotype, (ii) this protein should contain a higher than average number of Met residues and (iii) this protein should be conserved throughout evolution (like MsrA and MsrB). The SRP component Ffh fitted all of these criteria, and its conserved role in one of the most fundamental process of cellular life rendered it even more attractive.

SRP catalyzes protein targeting in all cellular organisms (Herskovits *et al*, 2000; Keenan *et al*, 2001). The eukaryotic SRP recognizes the NH_2-terminal signal sequence of targeted proteins as they emerge from the ribosome. Binding to the SRP arrests polypeptide elongation and mediates docking of the translating ribosome with receptors in the membrane, where translation resumes along with translocation to the lumen of endoplasmic reticulum. In *E. coli*, the SRP component is essential for viability and efficient protein integration in the inner membrane (Brown and Fournier, 1984; Phillips and Silhavy, 1992; Luirink *et al*, 1994; Ulbrandt *et al*, 1997). The *E. coli* SRP consists of the Ffh protein, the *ffs*-encoded 4.5S RNA and the FtsY receptor, which are the prokaryotic counterparts of the eukaryotic SRP54, 7SL RNA and SRα components, respectively. Functional conservation between eukaryotic and prokaryotic SRP components is best illustrated by the fact that human SRP54 can interact with bacterium 4.5S RNA, while Ffh substitutes for SRP54 (Poritz *et al*, 1990). A salient feature of all SRP54 homologs

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is the occurrence of a Met-rich domain, referred to as M-domain. A decade ago, in the so-called 'bristles' hypothesis, lateral chains of Met residues were proposed to have a particular role in signal peptide binding (Bernstein *et al*, 1989). Subsequently, the M domain was shown to contain the 4.5S RNA-binding site and to be required for binding to signal sequences of presecretory proteins (Römisch *et al*, 1990; Zopf *et al*, 1990). Structural analysis of the *Thermus aquaticus* Ffh revealed that the M domain exhibits a deep groove composed of hydrophobic amino acids, including Met residues (Keenan *et al*, 1998). This groove was proposed to be the signal sequence-binding pocket of SRP. The position of Met residues of the *E. coli* M domain could be inferred from the *T. aquaticus* structure. Likewise, Met residues were proposed to line the hydrophobic groove such that they could interact with presecretory proteins (Keenan *et al*, 1998). Moreover, a detailed structural analysis of the *E. coli* M domain–RNA complex was carried out (Batey *et al*, 2000). Noticeably, the Met382 residue that is invariant throughout all prokaryotic and eukaryotic sequences was found to locate within the RNA-binding region of the protein.

In this study, we report that in *E. coli* MsrA and MsrB enzymes are necessary for protecting SRP from oxidative damages. This finding establishes a functional link between oxidative stress and protein targeting.

Results

Oxidized Ffh is a substrate of MsrA and MsrB

We first tested the hypothesis that oxidation could convert Ffh to a bona fide substrate for MsrA and/or MsrB enzymes. Purified Ffh was submitted to oxidation by H_2O_2, and subsequently used as a substrate for MsrA and/or MsrB enzymes. Kinetic analyses revealed that MsrA and MsrB exhibited initial rates of NADPH oxidation of 6.4 and 5.35 μM of NADPH oxidized/min, respectively (Figure 1). When MsrA-catalyzed reaction reached completion, MsrB was added and the reaction was found to resume (Figure 1). Similarly, when MsrB-catalyzed reaction reached a plateau, addition of MsrA allowed it to resume (Figure 1). This was similar to what was previously found using oxidized calmodulin as a substrate, and pointed to the existence of two types of MetSO diastereoisomers in oxidized Ffh (Grimaud *et al*, 2001). When both MsrA and MsrB enzymes were added simultaneously, the initial rate of NADPH oxidation observed was 17.5 μM of NADPH oxidized/min (Figure 1). The total amount of NADPH oxidized with both MsrA and MsrB was the same as that reached when both enzymes were added sequentially (Figure 1). In this experiment, 10 μM Ffh was oxidized, yielding a concentration of MetSO residues in the reaction of 280 μM. As reaching the plateau needed 250 μM NADPH to be oxidized, this indicated that the oxidation protocol was efficient enough to convert most of the 28 Met residues in MetSO, an interpretation that fits with the mass spectrometry data described below (Figure 2). No Msr activity, was observed when nonoxidized SRP was used as a substrate (data not shown).

Mass spectrometry analyses of oxidized Ffh and of MsrA/B repaired Ffh

Purified Ffh was oxidized and submitted to repair by MsrA and MsrB. Samples containing native Ffh, oxidized Ffh or MsrA/B-repaired oxidized Ffh were run on SDS–polyacrylamide gel electrophoresis (PAGE). Bands of each sample were cut off the gel, submitted to trypsin digest and the resulting peptides were analyzed by MALDI/TOF-MS (Figure 2). The spectra obtained were scanned for Met-containing peptides. Trypsin hydrolysis was predicted to produce 11 peptides including at least one Met residue. Based on their predicted mass values, five could be identified within the spectra of tryptic digest of native Ffh. These peptides are visualized in the primary sequence shown in Figure 2A. Peptides 3 and 4 exhibited similar theoretical mass values and are expected to give rise to superimposed peaks (Figure 2B). When the spectrum of oxidized Ffh was scanned, mass values of peptides 2, 3, 4 and 5 were increased by 16 Da, corresponding to the gain of one oxygen atom (Figure 2C). No peaks were found at mass values corresponding to the reduced form of these peptides. Two peaks at mass values corresponding, respectively, to reduced and oxidized forms of peptide 1 were visible in the spectrum of oxidized Ffh (Figure 2C). The spectra of MsrA/B-repaired Ffh were found to contain only reduced forms of peptides 1, 3, 4 and 5 (Figure 2C). Both reduced and oxidized forms of peptide 2 were present (Figure 2C). Taken together, these analyses strongly suggested that oxidized Ffh contained MetSO residues that had been converted to Met in the MsrA/B-repaired Ffh.

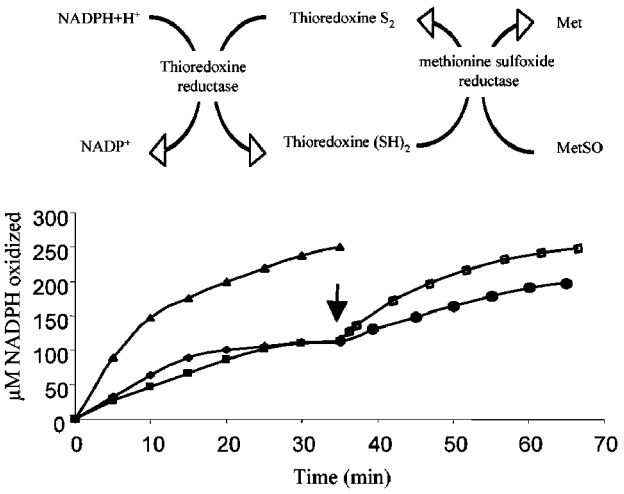
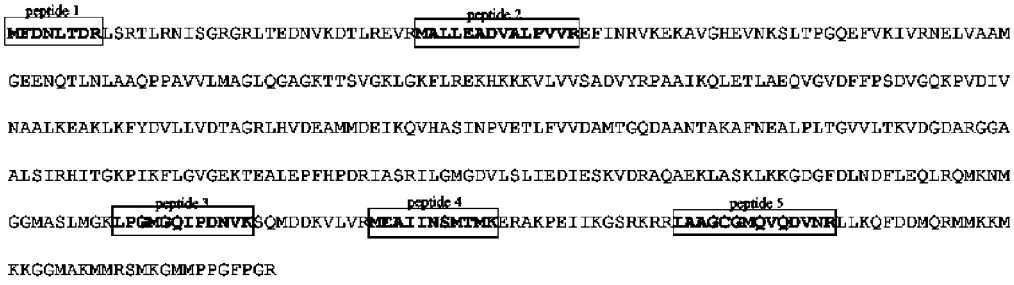


Figure 1 Oxidized Ffh is a substrate for MsrA and MsrB. Oxidized Ffh was obtained after submitting SRP to H_2O_2 (50 mM) as described in *Materials and methods*. The reaction mixtures included NADPH (400 μM), thioredoxin (5 μM), thioredoxin reductase (87 nM) and Ffhox (10 μM). In the first experiment, reaction was carried out first with 1 μM of MsrB (■) until completion, at which point (indicated by an arrow) 1 μM MsrA (□) was added. In the second experiment, reaction was carried out first with 1 μM of MsrA (♦) until completion, at which point 1 μM MsrB (●) was added. In the third experiment, both MsrA (1 μM) and MsrB (1 μM) were added simultaneously (▲). A schematic representation of the reaction catalyzed by Msr enzymes is shown above the curve.

Mass spectrometry analysis of the oxidation state of Ffh in msrA msrB mutants

We tested the effect of lack of MsrA and MsrB enzymes on the oxidation state of Ffh *in vivo*. Ffh-$(His)_6$ was overproduced in strains MC4100 and BE53, an isogenic derivative lacking both *msrA* and *msrB* genes. Total protein extracts were run on

A



B

Peptide number	Position	Peptide masses as $[M+H]^+$
1	1-8	1011.4564
2	36-49	1496.8505
3	354-365	1268.6667
4	376-386	1268.6047
5	402-415	1461.6937

C

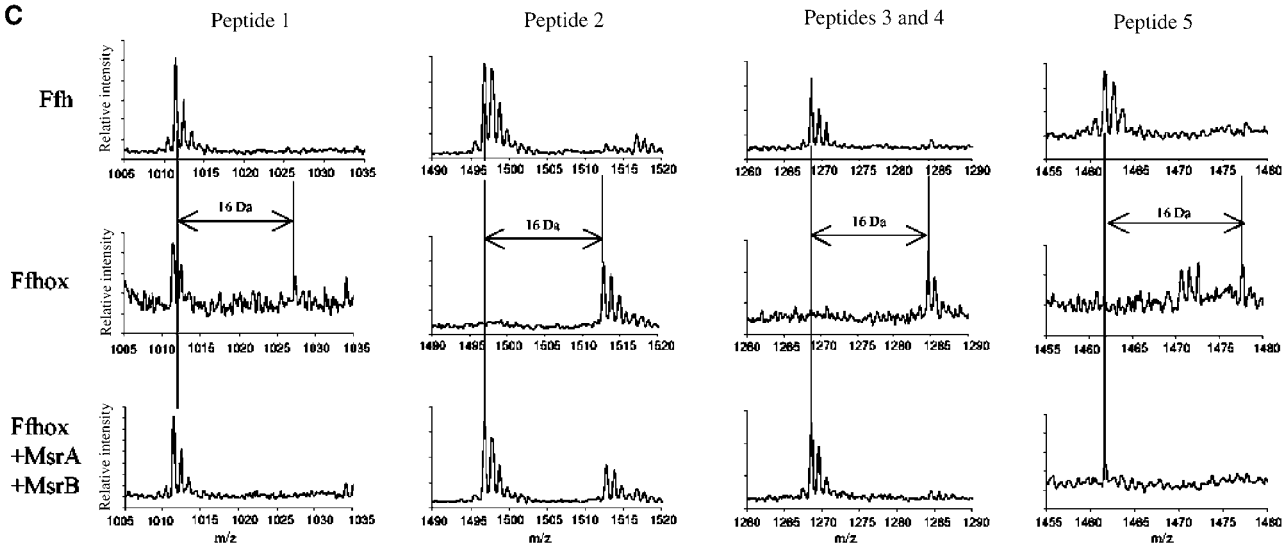


Figure 2 Oxidation and MsrA/B repair of Ffh. Pure Ffh was oxidized with H_2O_2 and subsequently repaired by MsrA and MsrB enzymes. Samples were then submitted to tryptic hydrolysis and analyzed by MALDI/TOF-MS. (**A**) The primary sequence of Ffh is shown. Peptides whose oxidation status was followed during the experiment are indicated in boxes. (**B**) Predicted mass values of peptides 1–5 described above are indicated. (**C**) Native Ffh, oxidized Ffh (referred to as Ffhox) and oxidized Ffh repaired with MsrA and MsrB were hydrolyzed and the resulting peptides were separated by MALDI/TOF-MS.

SDS–PAGE and bands corresponding to Ffh were excised, submitted to tryptic digestion and the resulting products were analyzed by mass spectrometry. The spectra of tryptic digest of Ffh synthesized in either background were scanned as described above. Peptides 1, 2, 3 and 4 could be identified. Both reduced and oxidized forms of peptides 2, 3 and 4 were to be found in MC4100 strains while, in contrast, only primarily forms were found in *msrA msrB* mutants (Figure 3). Both oxidized and reduced forms of peptide 1, which contains the initiator Met residue, were found in MC4100 and mutant strains (Figure 3). Taken together, these results suggested that *msrA msrB* mutation led to increased oxidation of Ffh. To ascertain that oxidation was due to aerobic metabolism, mass spectrometry analysis was then carried out using MC4100 and mutant strains grown under anaerobiosis. The vast majority of the three peptides analyzed was in a reduced form in the two strains (Figure 3).

Functional properties of oxidized Ffh and of MsrA/B-repaired Ffh

We investigated whether oxidized Ffh was able to interact with 4.5S RNA. *In vitro* synthesized 4.5S RNA was labelled with ^{32}P and incubated with increasing concentration of oxidized Ffh (Figure 4). Ffh/4.5S RNA interaction was investigated using a filter-binding assay as described by Suzuma *et al* (1999). At high concentrations of oxidized Ffh

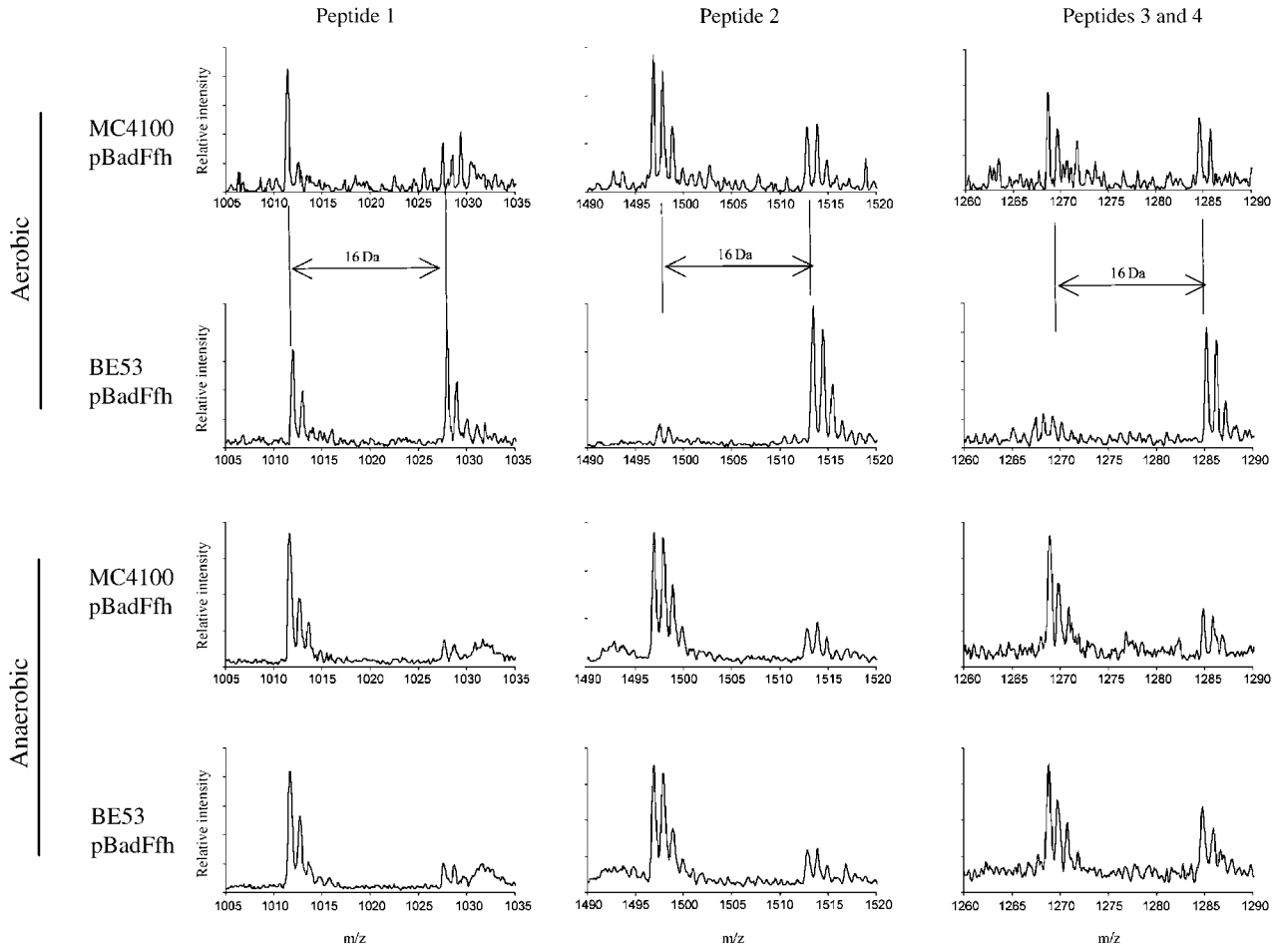


Figure 3 Oxidation status of Ffh synthesized in *msrA msrB* mutant of *E. coli*. A similar protocol as described in the legend of Figure 2 was followed, except that Ffh was produced *in vivo* using pBadFfh, either in MC4100 or in isogenic BE53 *msrA msrB* mutant. Experiments were carried out using either aerobic or anaerobic culture conditions. Cell extracts were run on SDS–PAGE and the band corresponding to Ffh was cut off the gel prior trypsin hydrolysis and MALDI/TOF-MS analysis.

(referred to as Ffhox) (10 μM), only 22.5% of added RNA was retained on the filter. In contrast, 0.5 μM of native Ffh was sufficient to retain over 90% added RNA. This result indicated that oxidized Ffh was altered in its ability to interact with 4.5S RNA.

Oxidized Ffh was submitted to repair by MsrA and MsrB and tested for its ability to bind 4.5S RNA (Figure 4). Using 10 μM of MsrA/B-treated oxidized Ffh allowed to retain 73% of added RNA. This showed that repaired Ffh recovered nearly native RNA-binding ability. Comparison with oxidized Ffh indicated that at a high concentration (10 μM), repaired Ffh was three times more efficient than oxidized Ffh in binding RNA. Interestingly, when oxidized Ffh was treated with either MsrA or MsrB alone, approximately 50% RNA was retained at high concentration (10 μM) (data not shown). This study demonstrated that the repair of oxidized Ffh by MsrA/B enzymes permits the recovery of its RNA-binding ability.

Stability of Ffh in msrA msrB mutant

Previous studies showed that Ffh is unstable *in vivo* when it is dissociated from 4.5S RNA (Jensen and Pedersen, 1994; Park *et al*, 2002). Therefore, a prediction of both *in vivo* and *in vitro* results is that the Ffh should be destabilized in *msrA msrB* mutants. Thus, MC4100 and BE53 (*msrA msrB*) strains were grown up to mid-exponential phase, submitted to chloramphenicol treatment, samples taken over time and analyzed by immunoblot for their content in Ffh. Ffh proved to be quite stable in MC4100 strain as ca. 80% of the initial amount was still present after 24 h incubation. In contrast, in BE53 strain, more than 50% of the initial Ffh was degraded in 6 h (Figure 5). These results indicated that a lack of Msr function resulted in destabilization of Ffh.

Genetic evidence for a physiological connection between MsrA, MsrB and Ffh

In *E. coli*, as the *ffh* gene is essential for cell viability, the steady state amount of Ffh must be kept above a threshold concentration. From the decreased stability of Ffh in *msrA msrB* mutants reported above followed the prediction that cell viability of *msrA msrB* mutants should require a higher level of Ffh synthesis as compared with the wild type. To test this prediction, we made use of the JM113 strain that contains a chromosomally located arabinose-inducible *ffh* gene (as unique *ffh* allele), allowing Ffh synthesis to be controlled. Both the JM113 strain and its isogenic derivative BEjm02

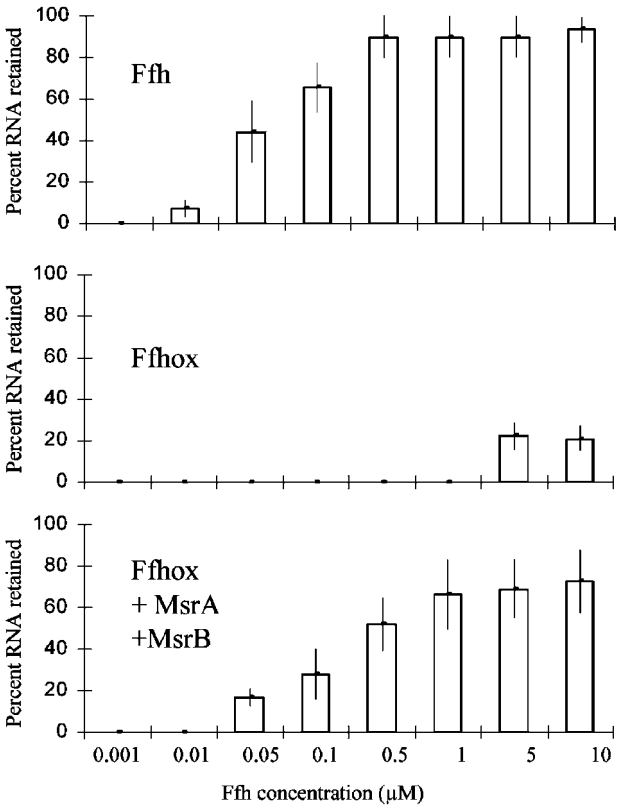


Figure 4 Interaction of 4.5S RNA with oxidized and repaired Ffh. Oxidized Ffh was obtained after submitting SRP to H_2O_2 (50 mM) as described in *Materials and methods*. A subset of it was incubated in the presence of both MsrA and MsrB in the presence of DTT. Radiolabelled 4.5S RNA was obtained by *in vitro* transcription of the *ffs* gene in the presence of [α-^{32}P]CTP. Nitrocellulose filter-binding assay was used to examine the 4.5S RNA (0.15 nM) binding activity of Ffh, Ffhox or MsrA/MsrB-repaired Ffhox, as indicated in the graphs. Ffhox stands for oxidized Ffh.

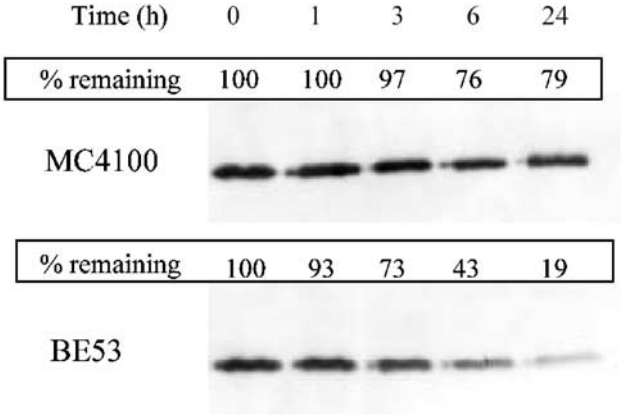


Figure 5 Importance of *msrA msrB* for the stability of Ffh *in vivo*. Cultures of *E. coli* MC4100 and BE53 (*msrA msrB*) were grown at 37°C until exponential phase, and chloramphenicol was added to stop synthesis. Aliquots were removed after 1, 3, 6 and 24 h and analyzed by SDS–PAGE, as indicated above the picture. Protein levels were analyzed by Western blotting by using an anti-Ffh antiserum. ECL film was scanned and band intensities were quantified. The percent of remaining Ffh is given above the protein bands in the picture.

(*msrA msrB*) grew normally when cultures were fully induced with 0.2% arabinose, that is, synthesizing large amounts of Ffh (Figure 6A). In contrast, when Ffh synthesis

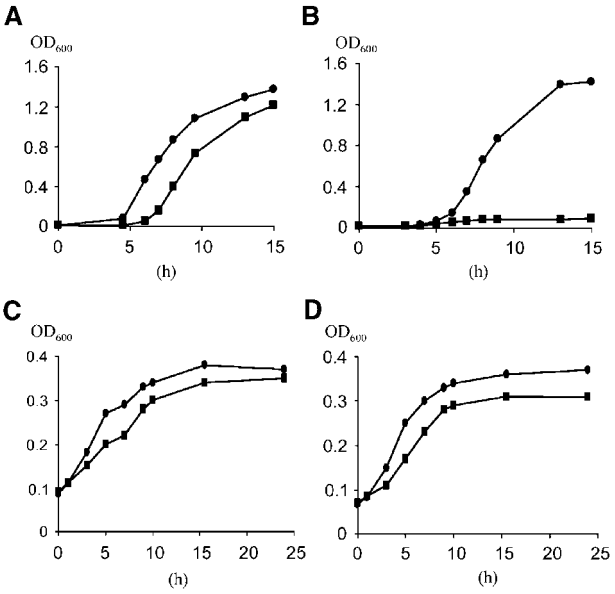


Figure 6 Synthetic lethality between the limiting amount of Ffh and *msrA msrB* mutations. Cultures of *E. coli* JM113 (*λara-ffh*) (●) and BEjm02 (*λara-ffh msrA msrB*) (■) carrying the pBR322 plasmid were grown in M9 medium with casamino acids supplemented with arabinose 0.2% (**A**) or arabinose 0.02% (**B**). OD_{600} values were recorded. It is important to note that concentrations of arabinose used were found by Siegele and Hue to allow induction of all cells in the population (Siegele and Hu, 1997). Experiments shown in (**C**) and (**D**) are identical to (A) and (B), respectively, with strains grown in anaerobiosis using trimethylamine oxide as an electron acceptor.

was reduced, that is, in the presence of 0.02% arabinose, no growth was observed for the BEjm02 strain, whereas JM113 strain grew normally (Figure 6B). This fitted the prediction that more Ffh needs to be synthesized in *msrA msrB* backgrounds as compared with JM113. Presumably, the higher demand in Ffh synthesis was related to increased oxidative damage in *msrA msrB* mutant. To validate this, a similar growth experiment was carried out in the absence of oxygen. Strains were grown under anaerobiosis with trimethyl amine as an electron acceptor. No difference of Ffh synthesis requirement was found between JM113 and BEjm02 strains, as they grew the same at both high and low arabinose concentrations (Figure 6C and D).

Importance of MsrA/B for in vivo SRP-dependent protein targeting

To examine the phenotypic consequences of *msrA msrB* mutations on Ffh-dependent targeting of proteins, the polytopic MalF protein was used as a model. Two MalF–PSBT hybrids previously studied by Tian *et al* (2000) were used. Hybrid J contains a biotinylation domain that upon membrane insertion locates in the periplasmic compartment, and hybrid K contains a cytoplasmic biotinylation domain. Biotinylation takes place exclusively in the cytoplasm and only biotinylated proteins can interact with streptavidin. Therefore, a hybrid protein will be recognized by streptavidin only if its biotinylation domain localizes in the cytosol. In the MC4100 cells, hybrid J failed to be recognized by streptavidin (Figure 7A). In contrast, inactivation of both *msrA* and *msrB* genes resulted in hybrid J biotinylation. Providing *msrA* or

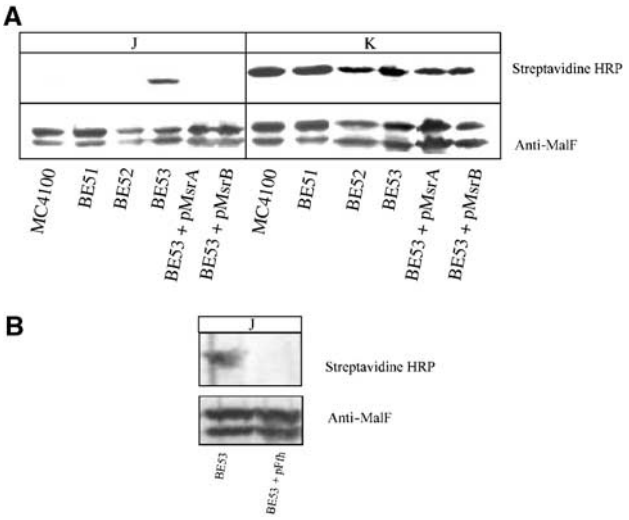


Figure 7 Importance of *msrA msrB* for proper membrane protein integration. (**A**) MalF–PSBT fusions J and K were synthesized in MC4100, BE51 (*msrA*), BE52 (*msrB*), BE53 (*msrA msrB*), BE53/pMsrA and BE53/pMsrB strains. Cells were grown at 37°C in M9 medium supplemented with 2 nM biotin and kanamycin (100 μg/ml). Synthesis of MalF–PSBT fusions was induced with 0.5 mM of IPTG, at an OD_{600} or 0.3. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. To detect biotinylated fusion proteins, streptavidin–horseradish peroxidase conjugate (Amersham Pharmacia) was used. The synthesized fusion proteins were detected using polyclonal anti-MalF. (**B**) Overproduction of Ffh suppresses translocation defects in *msrA msrB* mutant. MalF–PSBT fusion J was synthesized in BE53 (*msrA msrB*) and BE53/pFfh strains and studied as described above.

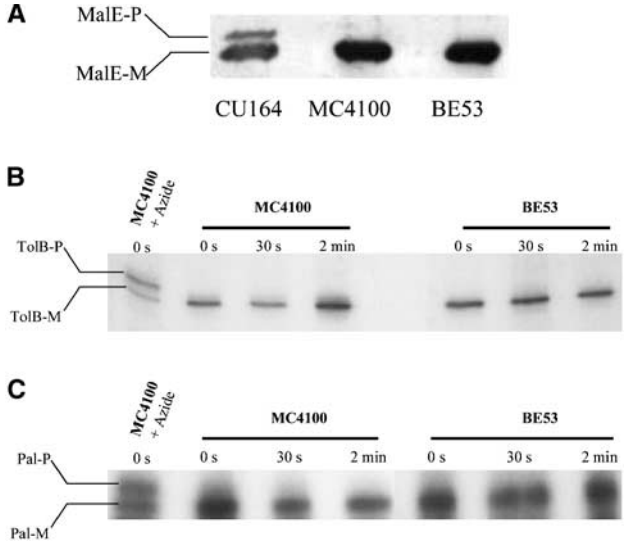


Figure 8 Sec-dependent translocation is unaffected by *msrA msrB* mutation. (**A**) Study of translocation of MalE. Cultures of MC4100, BE53 and CU164 were grown in M9 medium in the presence of maltose at 37°C. Cell extracts were prepared and analyzed by immunoblot using anti-MalE antiserum. A MalE precursor form was visible in *secY* background (indicated as MalE-P), while only mature forms were visible in MC4100 and BE53 strains. (**B**) Study of translocation of TolB. The fate of TolB was investigated by pulse-chase experiment. Use of sodium azide-treated cultures allowed to visualize the precursor form (lane 1). Pulse was carried out for 5 min, while samples were taken out at time points 0 s, 30 s and 2 min during the chase period and immunoprecipitated using anti-TolB antibodies. (**C**) Study of translocation of Pal. The fate of Pal was investigated by pulse-chase experiment using the same protocol as above, using anti-Pal antibodies.

msrB gene *in trans* allowed the recovery of correct membrane insertion as the J hybrid was no longer biotinylated (Figure 7A). This indicated that the presence of either one of the two Msr enzymes is sufficient to confer an MC4100 integration. This was consistent with the fact that single *msrA* or *msrB* mutation did not alter membrane insertion of J hybrid. Moreover, providing *ffh* gene *in trans* in *msrA msrB* backgrounds suppressed mislocation of MalF-PBST J hybrid, as this was later no longer biotinylated (Figure 7B). This confirmed that mislocation of J hybrid in *msrA msrB* strains was due to limitation in functional Ffh. As expected, the biotinylation pattern of hybrid K was found to be altered in none of the genetic backgrounds as expected. Immunoblot analysis using anti-MalF antibodies showed that similar amounts of J and K hybrid proteins were present in all strains used. Significantly, J hybrid was properly targeted in *wt* and in *msrA msrB* strains grown under anaerobiosis, indicating that oxygen was required for targeting defects to be seen (data not shown).

In order to test whether *msrA msrB* mutations had a general defect in protein translocation, the efficiency of the Sec pathway was investigated. The fate of three extracytoplasmic proteins was analyzed, namely, the periplasmic proteins MalE and TolB and the outer membrane lipoprotein Pal. Precursor forms of MalE were visible in the *secY* mutant (Figure 8A). In contrast, only mature forms of MalE were observed in wild type and in *msrA msrB* mutants, indicating that MalE had crossed the inner membrane equally efficiently in both strains. Similarly, pulse-chase experiments showed that translocation of neither TolB nor Pal was affected in the double mutant (Figure 8B and C).

Discussion

Ubiquitous methionine sulfoxide reductases MsrA and MsrB were proposed to be important for cells to resist oxidative stress (Weissbach *et al*, 2002). Here, we indeed demonstrate that MsrA and B are required to repair oxidatively damaged Ffh, a Met-rich protein conserved throughout procaryotes and eukaryotes. Hence, this study demonstrates that SRP-dependent protein translocation is a target for oxidation, and identifies the MsrA/B-repair pathway as a key actor in protecting this central cellular process.

Our aim was to identify cellular processes, the functioning of which relies on the MsrA/B repair pathway. We chose the SRP component Ffh as a model as it exhibits several criteria of interest (see Introduction), among which is a phylogenic ubiquity similar to that of Msr enzymes. In the first step, a series of chemical and biological studies were carried out *in vitro* to characterize the effects of oxidation on purified Ffh. Likewise, oxidized Ffh was found to contain MetSO residues and, accordingly, oxidation was shown to convert Ffh to a substrate for MsrA and MsrB enzymes. Subsequently, MetSO-containing Ffh was found to be unable to interact with 4.5S RNA and to form the SRP complex, while MsrA/B activities allowed oxidized Ffh to recover 4.5S RNA-binding abilities. This established the key role of MsrA/B in repairing oxidized Ffh. A second series of experiments were designed to investigate the *in vivo* situation. Mass spectrometry analysis of tryptic digests showed that Ffh synthesized in aerobically grown *E. coli msrA msrB* mutants contained MetSO residues

as well. This clearly indicated that lack of MsrA/B leads to accumulation of unrepaired, MetSO-containing Ffh, leading to the prediction that no SRP complex would be formed. Two lines of evidences supported this prediction. First, Ffh was found to be unstable in *msrA msrB* mutants, as previously observed with 4.5S RNA free Ffh. Second, SRP-dependent targeting was found to be hampered in the absence of *msrA msrB*-encoded enzymes, whereas Sec-dependent targeting was not. Moreover, a case of synthetic lethality was found when combining the reduced level of Ffh and inactivation of *msrA msrB* genes, giving further credence to a physiological connection between SRP-dependent protein translocation and MsrA/B repair pathway. Thus, both *in vivo* and *in vitro* studies converged to identify SRP-mediated protein targeting as a cellular target for MsrA/B repair.

Reduced virulence was found to be due to *msrA* mutation in several pathogens. Surprisingly, lack of MsrA enzyme led to defects in cell envelope-related process, that is, decreased type I fimbriae-mediated hemagglutination in enteropathogenic *E. coli* and loss of motility in *Erwinia chrysanthemi* (Wizemann *et al*, 1996; Hassouni *et al*, 1999). MsrA being cytoplasmic, the link with extracytoplasmic functions was unclear. A possibility was that these extracytosolic processes depend upon a cytosolic component, itself dependent upon a functional MsrA enzyme. Our present finding opens a new possibility. Adherence and cell motility depend on inner membrane proteins, the proper membrane integration of which is likely to depend upon SRP pathway. As a working hypothesis, we might envision that host-released ROS alter, among other cellular processes, SRP-dependent protein export. This could in turn reduce the targeting efficiency of virulence-associated factors. Hence, according to this scenario, *msrA* mutant would exhibit reduced virulence as it could not fully repair SRP. Against this view stands our own finding that *msrA* mutation alone does not alter SRP targeting and, hence, is unlikely to affect virulence. However, one should consider the fact that host-released ROS are likely to be in much higher amount than those produced by aerobic metabolism. The need for MsrAB repair might be more important in a host context than in a simple lab culture.

In vitro analysis established that both MsrA and MsrB enzymes differ in their diastereospecificity (Sharov *et al*, 1999; Kryukov *et al*, 2002; Olry *et al*, 2002). Yet, under normal laboratory growth conditions, mutating either *msrA* or *msrB* was neutral, suggesting that lack of one enzyme species could be compensated for by the presence of the other. In order to account for the neutral effect of mutations in either *msrA* or *msrB*, one must first assume that oxidation is essentially a stochastic process, and then, take into account the chemical and functional heterogeneity within a polypeptide population exposed to ROS. For each protein species, ROS exposure should yield a set of polypeptide chains that contain Met residues left nonoxidized, as well as both *S*- and *R*-MetSO diastereoisomers, the oxidized status of each Met residue varying from one polypeptide chain to the other. This assumption received experimental support in the present work, as a mixed population of reduced and oxidized forms was observed by mass spectrometry analysis of tryptic digests of *in vivo* synthesized Ffh. The fate of each polypeptide chain will then depend on the role of each Met residue: oxidation of Met residues located at the surface might make no difference in terms of stability and/or activity, whereas oxidation of structurally or functionally important Met might be harmful. When bacteria face low level of ROS produced endogenously by aerobic metabolism, the size of the oxidized subpopulation might be low and repair by either one of the two, MsrA or MsrB, might be sufficient. This could explain why *E. coli* strain synthesizing only one Msr exhibits a neutral phenotype under normal growth conditions. Alternatively, biochemical experiments have argued for the existence of an epimerase that could convert Met-*R*-SO to Met-*S*-SO (Weissbach *et al*, 2002). This could account for the neutral phenotype of *msrB* mutation at least.

If one assumes oxidation to be a stochastic process, the prediction is that the composition of a heterogenous polypeptide population will vary as a function of the ratio between the size of this population and the level of ROS. Experimental validation of this hypothesis was obtained by varying the size of the Ffh population and keeping the level of ROS constant. Thus, under aerobic conditions, we observed synthetic lethality between *msrA msrB* mutations and reduced Ffh synthesis. This indicated that oxidized Ffh accumulated in the absence of MsrA/B repair function, diminishing the size of the functional Ffh subpopulation. (A complementary evidence was provided by the fact that overproduction of Ffh could suppress MalF targeting defects of *msrA msrB* strain). Remarkably, when strains were grown in the absence of oxygen, synthetic lethality between reduced Ffh synthesis and *msrA msrB* mutant disappeared, demonstrating that oxidation was indeed the cause of the shortage in functional Ffh.

Materials and methods

Chemicals

Unless noted otherwise, chemicals were purchased from Sigma. Thioredoxin reductase was a gift from Dr C Williams (University of Michigan, Ann Arbor). Bacteria were grown on Luria-Bertani medium or M9 minimal medium.

Strain construction

The DNA region encompassing *msrA* gene was cloned into pUC18 plasmid. The resulting plasmid was restricted by *Hinc*II enzyme and ligated with a *Sma*I restricted fragment containing the ΩSpc cassette. The recombinant plasmid was then linearized and electroporated into *E. coli* KM354 (*recJ*) strain carrying the pTP223 plasmid (*bet gam exo*) (Murphy, 1998). Spc^R colonies were selected and checked for Amp^S phenotype. Polymerase chain reaction (PCR) was then used to check whether recombination occurred at the *msrA* locus. An *msrB* deletion-containing strain was constructed by replacing *msrB* gene with a Cam^R cassette from plasmid pKD3 in strain BW25113/pKD46, as previously described (Datsenko and Wanner, 2000). Mutations were subsequently transferred into a different strain by transduction with P1 phage (Table I).

Plasmid construction

The *ffh* coding region was amplified by PCR using chromosomal DNA from *E. coli* strain MG1655 as a template and the oligonucleotides pffhC (5′-GGTACGATCGCTCGAGGCGACCAGGGA AGCCTGGGGG-3′) and pffhN (5′-CTGATAGAATTCCATATGTTTGAT AATTTAACCG-3′) as primers. The PCR product was inserted into *Nde*I- and *Xho*I-restricted plasmid pET21a (Novagen), yielding plasmid pETffh.

The *ffh* coding region was amplified from chromosomal DNA from *E. coli* strain MG1655, using oligonucleotides 5′ coding pffh5, containing an *Eco*RI site (5′-CTGATAGAATTCTGGGCATGAAGGTTG CCA-3′) and 3′ reverse complement pffh3 containing an *Sph*I site (5′-TAGCTCAGCATGCGCAAACAAGCCAGGCCGA-3′). Note that pffh5 and pffh3 oligonucleotides are located at 520 nt upstream and 10 nt downstream of the *ffh* coding region. The resulting PCR

Table I Strains and plasmids

	Relevant characteristics	Reference or source
Strains		
MG1655	Wild type	Lab collection
JM113	*ffh*::Kan^R λ(*ara*$_p$-*ffh*, Amp^R)	MacFarlane and Muller (1995)
BEjm00	JM113 *msrA*::Spc^R	This study
BEjm01	JM113 Δ*msrB*::Cam^R	This study
BEjm02	JM113 *msrA*::Spc^R Δ*msrB*::Cam^R	This study
MC4100	*araD*139 Δ(*argF-lac*)U169 *rpsL*150 *rel*A1 *thi fib*B5301 *deo*C1 *pts*F25 *rbs*R	Lab collection
BE51	MC4100 *msrA*::Spc^R	This study
BE52	MC4100 Δ*msrB*::Cam^R	This study
BE53	MC4100 *msrA*::Spc^R Δ*msrB*::Cam^R	This study
CU164	*secY*39cs, *zhd*-33::Tn10	LF Wu
Plasmids		
pET21a	Amp^R	Lab collection
pETffh	pET21a carrying *ffh* gene	This study
pFfh	pUC18 carrying *ffh* gene	This study
pGEMT	Amp^R	Lab collection
pGEMTffs	pGEMT carrying *ffs* gene	This study

product was cloned into pUC18 plasmid, previously cut with *Eco*RI and *Sph*I enzymes, yielding pFfh.

Plasmid pGEMTffs was obtained by cloning *ffs* gene in the pGEMT vector (Promega). The *ffs* coding region was amplified by PCR using chromosomal DNA from *E. coli* strain MG1655 as a template and the oligonucleotides pffs5′ (5′-TGACGTCGCGTTGGTT CTCAACGC-3′) and pffs3′ (5′-TATGCATGGGTGGGGGCCCTGCCAG -3′) as primers. The PCR product was inserted in *Aat*II–*Nsi*I restricted pGEMT plasmid, yielding plasmid pGEMTffs.

Purification of Ffh, MsrA and MsrB proteins

E. coli strain BL21 (DE3)/pETffh cells were grown at 37°C in Luria Bertani containing 100 μg/ml ampicillin. At an OD_{600} of 0.6, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added, and growth was continued for 2 h. Cells were harvested by centrifugation. The pellet (1.8 g) was resuspended in 7.2 ml of buffer A (25 mM Hepes, pH 7.5, 0.3 M KCl, 10% (v/v) glycerol). Resuspended cells were broken by a single pass through an ice-chilled French pressure cell at 6 tons. The resulting crude extract was centrifuged at 30 000 *g* for 30 min at 4°C. The supernatant was applied onto a 5 ml Hi-trap column (Amersham Pharmacia Biotech) loaded with nickel and equilibrated with buffer A plus 0.05 M imidazole. Proteins were eluted with a 13 ml gradient imidazole (0.05–0.5 M). Fractions were analyzed by SDS–PAGE. After elution with imidazol, the Ffh-containing fractions were pooled, and the solution was diluted to reach a KCl concentration of 0.1 M. This solution was loaded onto a MonoQ column ($0.5 \times 5\,cm^2$) (Amersham), equilibrated with buffer B (same as buffer A with 0.1 M KCl). Ffh was eluted with 20 ml gradient ranging from 0.1 to 0.7 M KCl. Spectrophotometric and ethidium bromide-stained agarose gel analyses showed that purified Ffh was 4.5S RNA-free.

MsrA and MsrB proteins were purified by column chromatography as previously described (Grimaud *et al*, 2001).

In vitro oxidation of Ffh

Ffh (10 mg in buffer B described above) was treated with 50 mM H_2O_2 for 4 h at room temperature, as described by Levine *et al* (1996). H_2O_2 was removed by gel filtration through G25 Sephadex. The released oxidized Ffh was then concentrated by ultrafiltration on ultrafree biomax-5 K (Millipore).

Methionine sulfoxide reductase activity assay

Methionine sulfoxide reductase activity was assayed in buffer A (25 mM Hepes, pH 7.5, 0.3 M KCl, 10% (v/v) glycerol) as described previously (Moskovitz *et al*, 1996; Grimaud *et al*, 2001).

Nitrocellulose filter-binding assay

4.5S RNA was obtained by *in vitro* run-off T7-mediated transcription of plasmid pGEMTffs after cleavage with *Nsi*I restriction endonuclease. ^{32}P-labelled *ffs* (0.15 nM) was incubated with various concentrations of different forms of Ffh, that is, oxidized Ffh (Ffhox), MsrA-treated Ffhox, MsrB-treated Ffhox and MsrA/MsrB-treated Ffhox, in 10 μl of a buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 300 mM KCl, 1 unit poly (dIdC) for 1 min at room temperature. Mixtures were then diluted with 0.5 ml of wash buffer (20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 100 mM NaCl, 10% glycerol). The resulting solutions were immediately passed through nitrocellulose filters (Millipore 0.45 μm HA), which were washed with 1.5 ml of wash buffer as described previously (Suzuma *et al*, 1999). The levels of radioactivity remaining on the nitrocellulose filters were determined by a liquid scintillation analyser (Packard 2200CA).

Preparation and detection of biotinylated fusion proteins

Detection of biotinylation fusion proteins was carried out as described previously (Tian *et al*, 2000). Cells were grown overnight at 37°C in M9 medium with appropriate antibiotics. Overnight cultures were spun down, resuspended in the same medium supplemented with 2 nM biotin and kanamycin (100 μg/ml). The MalF–PSBT fusion was induced with 0.5 mM of IPTG, at an OD_{600} of 0.3. Whole-cell proteins were precipitated after cells reached an OD_{600} of 0.6 with 6% trichloroacetic acid and resuspended in SDS sample buffer. After electrophoresis, proteins were transferred to a nitrocellulose membrane. To detect biotinylated fusion proteins, streptavidin–horseradish peroxidase conjugate (Amersham Pharmacia) was used. To detect the expressed fusion protein, we used polyclonal anti-MalF-PhoA J (a gift from Jon Beckwith). The ECL system (Amersham Pharmacia) was used for the subsequent detection following the manufacturer's instruction.

Pulse-chase experiments and immunoprecipitation

MC4100 and BE53 carrying the plasmid pFAIII (Isnard *et al*, 1994) were grown overnight at 37°C in minimal M9 medium added with tetracyclin, glucose (0.2%) and all amino acids, excluding Met and cysteine. Overnight cultures were used to inoculate the same medium (OD_{600} of 0.02). Cultures were grown with aeration at 37°C until an OD_{600} of 0.4 was attained. Aliquots (3 ml) were removed from each culture and labelled for 5 min with 50 μCi/ml of Redivue promix [^{35}S] Met. Then, 1.5 mg of cold Met was added and chased for various times (0 s, 30 s, 2 min). Whole-cell proteins were precipitated with 5% trichloroacetic acid. Immunoprecipitation of TolB and Pal was performed as described previously (Kumamoto and Gannon, 1988). Precursor forms were visualized by treating cultures with sodium azide (25 mM) for 5 min prior to the pulse.

Stability of Ffh in msrA msrB mutant

Cultures of *E. coli* MC4100 and BE53 (*msrA msrB*) were grown at 37°C in M9 medium with casamino acids. At an OD_{600} of 0.3,

aliquots (1 ml) were removed and chloramphenicol was added (2.5 mg/ml) to the remaining cultures. Aliquots (1 ml) were removed after 1, 3, 6 and 24 h and analyzed by SDS–PAGE. Protein levels were analyzed by Western blotting by using an anti-Ffh antiserum. The ECL film was scanned, and band intensities were quantified using Kodak image station 440CF.

MALDI/TOF-MS analysis of Ffh

Protein bands were excised from the gel, destained and digested with a solution of modified trypsin (10 ng/µl, sequence grade, Promega, Charbonnieres, France) in 25 mM NH_4HCO_3 (Shevchenko *et al*, 1996). The mixture was incubated at 37°C for 6 h. ZipTip pipette tips containing C_{18} reversed phase are used for desalting and concentrating peptides according to the manufacturer's instructions (Millipore corporation, Bedford, MA, USA) and eluted with 70% acetonitrile in water, 0.1% trifluoroacetic acid (v/v). Peptide solutions were mixed with an equal volume of α-cyano-4-hydroxycinnamic acid matrix solution (Sigma, Saint Louis, MO, USA), 10 mg/ml 70% acetonitrile in water, 0.1% TFA and applied to a sample plate for MALDI-MS. Isotope ^{12}C masses were determined in the positive ion reflector mode with MALDI/TOF-MS spectrometer Voyager DE-RP (Applied Biosystems, CA, USA) and internal calibration.

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